

CLAIMS

What is claimed is:

1. A method of expressing an immunotoxin in *Pichia pastoris* comprising
 - a) growing a *Pichia pastoris* that expresses an immunotoxin in a growth medium comprising an enzymatic digest of protein and yeast extract; and
 - b) performing methanol induction of the *Pichia pastoris* ,wherein the methanol induction is at a temperature of below about 17.5°C.
2. The method of claim 1, wherein the methanol induction is a limited methanol feed of between 0.5-0.75 ml/min (per 10 L initial medium).
3. The method of claim 1, wherein the methanol induction is a methanol and glycerol containing feed.
4. The method of claim 3, wherein the ratio of methanol to glycerol in the methanol and glycerol containing feed is about 4:1.
5. The method of claim 1, wherein the immunotoxin is a fusion protein.
6. The method of claim 1, wherein the immunotoxin comprises a diphtheria toxin moiety.
7. The method of claim 6, wherein the diphtheria toxin moiety is truncated.
8. The method of claim 7, further comprising a CD3 antibody moiety.
9. The method of claim 8, wherein the immunotoxin comprises A-dmDT390-bisFv(G₄S).
10. The method of claim 1, wherein the *Pichia pastoris* comprises a mutation in the amino acid sequence encoding EF-2.

11. The method of claim 1, wherein the enzymatic digest of protein is an enzymatic digest of soy protein.
12. The method of claim 1, further comprising contacting *Pichia pastoris* with phenylmethanesulfonyl fluoride and a source of amino acids.
13. The method of claim 12, wherein the *Pichia pastoris* is contacted with the phenylmethanesulfonyl fluoride and the source of amino acids for at least 2 hours.
14. The method of claim 12, wherein the phenylmethanesulfonyl fluoride is dissolved in the 4:1 methanol glycerol induction feed and the concentration does not exceed 10 mM.
15. The method of claim 12, wherein the source of amino acids is a yeast extract.
16. The method of claim 1, wherein the temperature can be selected from the group of temperatures consisting of 17.5, 17.0, 16.5, 16.0, 15.5, 15.0, 14.5, 14.0, 13.5, 13.0, 12.5, and 12.0°C.
17. The method of claim 1, wherein the temperature is about 15°C.
18. The method of claim 1, wherein the composition of the growth medium is about 4% glycerol, about 2% yeast extract, about 2% enzymatic digest of soy protein, about 1.34% yeast nitrogen base with ammonium sulfate and without amino acids, and about 0.43% PTM1 solution.
19. The method of claim 18, wherein the growth medium further comprises an antifoaming agent.
20. The method of claim 19, wherein the antifoaming agent is at a concentration of about 0.01% or greater.
21. The method of claim 20, wherein the composition of the growth medium is about 4% glycerol, about 2% yeast extract, about 2% enzymatic digest of soy protein, about 1.34% yeast nitrogen base with ammonium sulfate and without amino acids, about 0.43% PTM1 solution and about 0.02% antifoaming agent.

22. The method of claim 1, wherein dissolved oxygen concentration in the growth medium is maintained at a value of 40% or higher.
23. The method of claim 1, wherein the growth step is at a pH of about 3.5 and the methanol induction step is at a pH of about 7.0.
24. The method of claim 1, wherein the methanol induction step is performed for between about 22 and 288 h.
25. A method of expressing an immunotoxin in *Pichia pastoris* comprising
 - a) growing a *Pichia pastoris* that expresses an immunotoxin in a growth medium comprising an enzymatic digest of protein and yeast extract;
 - b) performing methanol induction of the *Pichia pastoris*, wherein the methanol induction comprises a limited methanol feed of 0.5-0.75 ml/min/10L of initial volume, wherein the induction is performed at a temperature below 17.5 °C, wherein an antifoaming agent supplied up to 0.07%, wherein agitation is maintained at about 400 RPM, and wherein the induction step is performed for between about 22 and 288 h.
26. A method of expressing an immunotoxin in *Pichia pastoris* comprising
 - a) growing a *Pichia pastoris* that expresses an immunotoxin in a growth medium comprising about 4% glycerol, about 2% yeast extract, about 2% enzymatic digest of soy protein, about 1.34% yeast nitrogen base with ammonium sulfate and without amino acids, and about 0.43% PTM1 solution, wherein the growth occurs at a pH of about 3.5, and wherein the dissolved oxygen concentration in the growth medium is maintained at a value of 40% or higher; and
 - b) performing methanol induction of the *Pichia pastoris*, wherein the methanol induction comprises a limited methanol feed of 0.5-0.75 ml/min/10L of initial volume, wherein the induction is performed at a temperature is 15 °C, wherein the pH is about 7.0, wherein antifoaming

agent supplied at 0.02%, wherein the agitation is maintained at about 400 RPM, and wherein the induction step is performed for about 163 h.

27. A method of purifying a non-glycosylated immunotoxin comprising
- a) loading a solution containing the non-glycosylated immunotoxin onto a hydrophobic interaction column;
 - b) obtaining a first non-glycosylated immunotoxin containing eluant from the hydrophobic interaction column;
 - c) loading the non-glycosylated immunotoxin containing eluant from step (b) onto an anion exchange column;
 - d) obtaining a second non-glycosylated immunotoxin containing eluant from the anion exchange column by eluting the non-glycosylated immunotoxin with a sodium borate solution;
 - e) diluting the concentration of sodium borate in the second non-glycosylated immunotoxin containing eluant from step (d) to about 50 mM or less;
 - f) concentrating the diluted non-glycosylated immunotoxin containing eluant from step (e) over an anion exchange column; and
 - g) obtaining a purified non-glycosylated immunotoxin from the anion exchange column.
28. The method of claim 27, wherein the non-glycosylated immunotoxin is expressed in yeast.
29. The method of claim 28, wherein the yeast is *Pichia pastoris*.
30. The method of claim 27, wherein the immunotoxin is a fusion protein.
31. The method of claim 27, wherein the immunotoxin comprises a diphtheria toxin moiety.
32. The method of claim 31, wherein the diphtheria toxin moiety is truncated.

33. The method of claim 32, further comprising a CD3 antibody moiety.
34. The method of claim 33, wherein the non-glycosylated immunotoxin comprises A-dmDT390-bisFv(G₄S).
35. The method of claim 27, further comprising washing the anion exchange column with about 25 mM sodium borate solution prior to eluting with the sodium borate solution.
36. The method of claim 27, wherein the concentration of the sodium borate solution in step (d) is between about 50 mM and about 200 mM.
37. The method of claim 36, wherein the concentration of the sodium borate solution in step (d) is between about 75 mM and about 100 mM.
38. The method of claim 27, wherein the concentration of sodium borate in step (e) is about 20 mM.